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Investigating the free radical trapping ability of NXY-059, S-PBN and PBN

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Abstract
The spin trapping ability of the nitrones 2,4-disulphophenyl-*N*-*tert*-butyl nitrone (NXY-059), 2-sulphophenyl-*N*-*tert*-butyl nitrone (S-PBN) and α-phenyl-*N*-*tert*-butyl nitrone (PBN) for both hydroxyl and methanol radicals was investigated using electron paramagnetic resonance (EPR) spectroscopy. The radicals of interest were generated *in situ* in the spectrometer under constant flow conditions in the presence of each nitrone. The spin adducts formed were detected by EPR spectroscopy. This approach allowed for quantitative comparison of the EPR spectra of the spin adducts of each nitrone. The results obtained showed that NXY-059 trapped a greater number of hydroxyl and methanol radicals than the other two nitrones, under the conditions studied.

Keywords: *Spin-trapping, free radicals, nitrones, PBN, S-PBN, NXY-059*

Introduction

There is significant evidence supporting the contention that free radical production is a key factor in the development of brain injury following both cerebral ischemia and reperfusion [1,2]. The use of compounds which trap free radials to ameliorate ischemic damage is therefore a logical therapeutic approach [3–5]. The nitrone compound α-phenyl-*N*-*tert*-butyl nitrone (PBN) was first reported to have neuroprotective properties in reports from two unconnected laboratories [6,7] and since that time a substantial literature has emerged confirming its neuroprotective actions in diverse animal models of cerebral ischemia [3]. Two other nitrone-derived compounds, 2-sulphophenyl-*N*-*tert*-butyl nitrone (S-PBN) and 2,4-di sulphophenyl-*N*-*tert*-butyl nitrone (NXY-059), have also been reported to be very effective neuroprotective agents in animal models of stroke [3–5].

The neuroprotective effects of nitrones are assumed to occur via the trapping of detrimental free radical species. The approach of spin trapping has been used for nearly 40 years to aid the detection and characterization of transient free radical species by electron paramagnetic resonance (EPR) spectroscopy [8,9], also known as electron spin resonance (ESR) spectroscopy. Nitrone or nitroso compounds are typically used that react with otherwise unstable free radical species to form longer-lived spin adduct species. The spin adduct species can be detected by EPR spectroscopy and the resultant spectra interpreted to determine the type of free radical that has been trapped.

While several studies have examined the ability of PBN to trap radical species, only one study has

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examined the relative abilities of PBN, S-PBN and NXY-059 to trap radicals [10]. The study showed that the three nitrones trapped both carbon- and oxygen-based radicals (including the hydroxyl radical) to give spin adduct species that could be detected by EPR spectroscopy. Although NXY-059 gave the most intense radical adduct signal of the three nitrones, it could not be concluded that NXY-059 was the most effective compound because of the possibility that the EPR responses were affected not just by the amount of each spin adduct formed, but also by the stability of the spin adduct species, as the authors acknowledged [10]. Furthermore, the experimental conditions involved the use of ultraviolet light to produce the hydroxyl radical from the breakdown of hydrogen peroxide. Since NXY-059 readily undergoes photo-degradation under these conditions significant degradation of the compound and potentially the spin adduct may have occurred.

We have therefore undertaken a study of the three nitrones, using new experimental approaches allowing quantitative comparisons of spin trapping. The ability of the three nitrones to trap both the hydroxyl radical and methanol radical has been quantitatively determined.

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Methods and materials

Materials

Due to the hydroscopic nature of NXY-059, a trihydrate salt was used for this study to aid sample handling. NXY-059 (purity $>$ 98%) was supplied by AstraZeneca (Södertälje, Sweden). PBN (98%) and S-PBN (95%) were purchased from Sigma Aldrich, Dorset, UK.

For the EPR and NMR studies, hydrogen peroxide (27.5% in water), titanium chloride (15% solution in HCl) and iron chloride hydrate were used as received from Riedel-de Haën, Seelze, Germany. 2,2,6,6-tetramethylpiperdine-*N*-oxyl (TEMPO, 99%) and 5,5 dimethyl-1-pyrroline *N*-oxide (DMPO) were purchased from Sigma Aldrich and used as received for calibration of the EPR response and further spin trapping comparison studies, respectively. For the NMR studies, deuterium oxide was used as received from FluoroChem, Derbyshire, UK.

EPR measurements

In these studies the hydroxyl radical was generated using Fenton chemistry reaction *in situ* in the EPR spectrometer sample cavity. The EPR spectra were recorded at room temperature on a cw-Bruker EMX X-band spectrometer, operating at 100 kHz field modulation and equipped with a Bruker ER4117MX mixing cell resonator. This resonator allows two reactant solutions to be mixed *in situ* in the EPR spectrometer. Spectra were acquired using the following settings; 1.0 G modulation amplitude, 2.0 mW microwave power, 100 G field width, 1×10^5 receiver gain and a 21 s scan time. A syringe pump (Harvard Apparatus 22) was used to deliver the reactant solutions into the mixing cell resonator at a fixed flow rate (4 or 8 ml/min). In order to generate the hydroxyl radical in the presence of each spin trap under test, reactant solution A (0.275% v/v hydrogen peroxide and 2 mM spin trap in water) was mixed with reactant solution B (metal catalyst in water) using the mixing cell resonator. The study was repeated using two different metal catalysts; titanium chloride ($TiCl_3$) at 0.1% v/v with a resultant pH of reaction of 1.8 and 2 mM iron chloride ($FeCl_2H_2O$) with a resultant pH of 3.2. For competitive spin trap studies, reactant solution A was prepared such that it contained two of the spin traps under test at $\sim$2 mM. Methanol radical trapping experiments were performed at pH 1.8 using titanium chloride as described above but in the presence of 1% v/v methanol.

To aid interpretation and to determine the hyperfine splitting constants A_N and A_H all the spectra obtained were simulated using the s*imEPR32* program, version 0.022alfa [11]. All experiments were performed in triplicate and the standard deviation of the results calculated. Quantitative results were obtained by performing the double integral of each spectrum using the WINEPR program, version 2.11 (Bruker). The system was calibrated at both flow rates using a series of Tempo standards in water, prepared over the concentration range 1×10^{-7} to 3×10^{-5} M. In this way the double integral results obtained for each sample were converted to an equivalent Tempo molar concentration and as such to the number of spin adduct molecules detected per mole of spin trap present.

The lifetime of the hydroxyl radical spin adducts of each nitrone were investigated by monitoring the EPR response of the spin adduct when the syringe pump was switched off and the flow of reactant solutions halted. The decay of the low field peak of the six line spectrum of each adduct was followed by repeatedly acquiring EPR spectra using the following settings; 1.0 G modulation amplitude, 2.0 mW microwave power, 10 G field width and a 5.2 s scan time and monitoring the EPR peak height over time as the flow of reactant solutions was halted. Again the measurements were performed in triplicate.

NMR measurements

The spin trap under test ($\sim$30 mg), 20 µl hydrogen peroxide and 15 µl titanium chloride (15% solution in HCl) were diluted with 10 ml deuterium oxide. These conditions mimicked the ratio of components in the EPR experiments, although the actual concentration

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was higher; 1 ml of this was then analysed immediately by ^{1}H NMR in a Bruker DPX400 using a 45° pulse, 10 s relaxation delay and 256 scans (total experiment time 45 min). An approximate assessment of the degree of degradation was made by integration of the signals due to the spin trap under test vs. the degradation product. A sample of the spin trap (~30 mg) and 20 µl hydrogen peroxide were diluted with 10 ml deuterium oxide and also analysed as described above. These conditions mimicked reactant solution A. Assignment of NXY-059 and structural determination of the degradation products under the experimental conditions above were performed on a Bruker A600 spectrometer with a TCI cryoprobe using a combination of ^{1}H—^{13}C Heteronuclear Single Quantum Correlation and ^{1}H—^{15}N Heteronuclear Multiple Bonds Correlation experiments.

Results

Initially the EPR response for the hydroxyl adduct of each spin trap was monitored as a function of the syringe pump flow rate; the titanium chloride experimental conditions were used for this study. At low flow rates (less than 4 ml/min) the EPR response for all three nitrones appeared to be affected by the stability of the spin adducts, but as the flow rate was increased (i.e. the time within the EPR sample cavity decreased) the EPR response became independent of the flow rate, as shown in Figure 1. At flow rates of greater than 4 ml/min the EPR response for the hydroxyl spin adduct of each nitrone was therefore considered to be independent of the stability of the spin adduct species, allowing quantitative comparison of the results for the three nitrones. For the experiments involving titanium chloride, at pH 1.8, a flow rate of 8 ml/min was therefore chosen. When iron chloride was used as the catalyst the resultant pH of the *in situ* reaction was less acidic (pH 3.2) and the concentration of hydroxyl radicals formed was therefore lower than at pH 1.8. The flow rate therefore had to be reduced in the iron chloride experiments, to allow the length of time that the spin adduct species spent within the EPR sample cavity to be maximized and so give the optimum EPR response without the spin adduct stability becoming influential. A flow rate of 4 ml/min was therefore chosen for the studies performed using iron chloride.

Individual spin trapping studies

Hydroxyl radical and methanol radical spin adduct species were detected for all three nitrones under the experimental conditions used. The experiments were performed in triplicate for each nitrone and the resultant spectra simulated to accurately determine the hyperfine splitting constants, A_N and A_H. A typical spectrum obtained for the NXY-059-OH adduct, along with the simulated spectrum, is shown in Figure 2. The A_N and A_H values obtained and the quantitative results for the three nitrones are shown in Table I. In the case of the methanol radical trapping experiments, the methanol radical itself was sufficiently stable to be detected by EPR. In the presence of each spin trap the resultant spectrum therefore contained overlapping contributions from the spin adduct and from the free methanol radical. The ratio of the contribution of the two species was determined during the simulation and then used to calculate the quantitative results.

The A_N and A_H values obtained were in agreement with results previously published by Maples et al. [10] and for PBN comparable with results contained in the NIEHS spin trap database [9] (S-PBN and NXY-059 are not in the database). NXY-059 trapped 1.6×10^{19} hydroxyl radicals and 1.1×10^{19} methanol radicals at pH 1.8, compared to 1.1×10^{19} and 7.9×10^{18}, respectively, for S-PBN and 1.0×10^{19} and 4.6×10^{18}, respectively, for PBN. These results (Table I) suggest that NXY-059 is the most effective of the three nitrones for trapping both the hydroxyl and methanol radicals under the conditions studied and that the differences detected are statistically significant (greater than three times the standard deviation results).

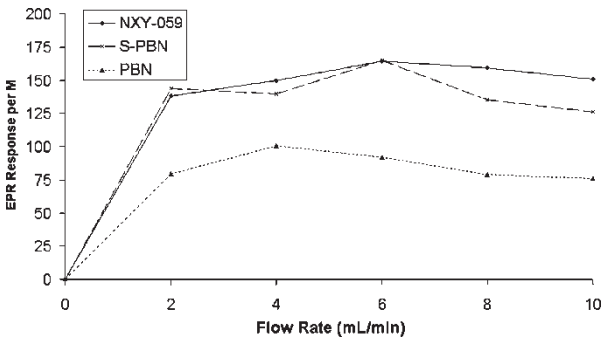

Figure 1. Change in EPR response (peak height) of hydroxyl spin adduct of the three nitrones as a function of the reactant solutions pump flow rate, to determine optimum experimental flow rate conditions.

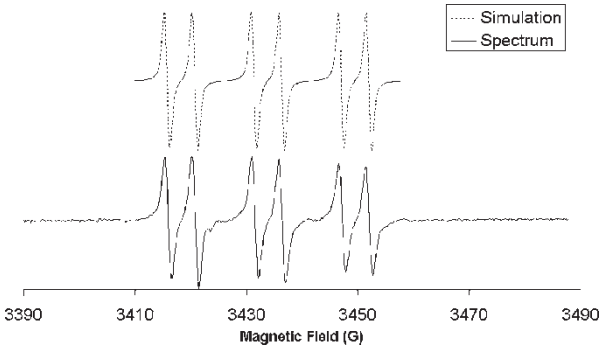

Figure 2. Typical EPR spectrum and simulation of the NXY-059 hydroxyl radical spin adduct. Instrument setting: receiver gain 1×10^{5}, microwave power 2 mW, modulation amplitude 1 G, scan time 21 s, 9 scans.

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Table I. Individual radical trapping results.

	NXY-059	S-PBN	PBN
Hydroxyl radicals			
Ti(III)—pH 1.8			
Spin adduct A_N (G) , M (SD)	15.61 (0.03)	15.73 (0.01)	15.51 (0.04)
Spin adduct A_H (G), M (SD)	4.89 (0.07)	5.10 (0.02)	2.55 (0.02)
Number of spin adduct molecules (SD)	1.64×10^{19} (1.50×10^{18})	1.08×10^{19} (4.65×10^{17})	1.00×10^{19} (3.40×10^{17})
Fe(II)—pH 3.2			
Spin adduct A_N (G), M (SD)	15.64 (0.03)	15.73 (0.02)	15.50 (0.02)
Spin adduct A_H (G), M (SD)	4.85 (0.01)	5.12 (0.01)	2.56 (0.02)
Number of spin adduct molecules (SD)	3.01×10^{18} (1.22×10^{17})	1.53×10^{18} (7.23×10^{16})	9.41×10^{17} (1.36×10^{17})
Methanol radicals			
Ti(III)—pH 1.8			
Spin adduct A_N (G), M (SD)	15.67 (0.03)	15.86 (0.02)	16.00 (0.001)
Spin adduct A_H (G), M (SD)	5.33 (0.03)	5.58 (0.03)	3.70 (0.03)
Number of spin adduct molecules (SD)	1.10×10^{19} (1.35×10^{17})	7.91×10^{18} (5.86×10^{17})	4.57×10^{18} (1.06×10^{17})

At pH 3.2, NXY-059 trapped almost twice the amount of radicals trapped by S-PBN (3.0×10^{18} and 1.5×10^{18}, respectively) and more than three times the amount trapped by PBN (0.9×10^{18}).

Competitive spin trapping studies

To directly compare the spin trap abilities of the three nitrones for the hydroxyl radical the experiments were repeated in the presence of two of the nitrones simultaneously. As shown in Table I the hydroxyl radical spin adducts of NXY-059 and PBN have significantly different A_H values, such that when both species are present the EPR spectrum can be interrogated to determine the percentage of each species present. The same is also true for PBN and S-PBN. The hyperfine coupling constants of the hydroxyl radical spin adducts of S-PBN and NXY-059 are similar with respect to the spectral line width and so the EPR spectra overlap, hindering interpretation. Studies were therefore performed at both pH values in the presence of NXY-059 and PBN simultaneously and then with PBN and S-PBN simultaneously. The typical spectra and simulations obtained are shown in Figure 3. Again the ratio of the contribution of the two species to the EPR spectrum was determined during the simulation and then used to calculate the quantitative results, shown in Table II. The A_N and A_H values obtained were comparable to those from the individual spin trap studies. Within experimental error similar results were obtained for the three nitrones in terms of the number of molecules of each spin adduct species detected at pH 3.2. At pH 1.8 PBN appeared to trap more hydroxyl radicals than both S-PBN and NXY-059. This change in spin trap ability with pH suggests that there may be issues with the stability of the three nitrones under the experimental conditions, especially at pH 1.8. Similar competitive studies were also performed for the methanol radical but the additional contribution to the spectrum from the methanol radical itself, as well as two spin adduct species, made the spectra difficult to interpret conclusively so no quantitative results were obtained.

Similar competitive hydroxyl radical trapping studies were also performed for each nitrone in the presence of DMPO, using iron chloride as the catalyst. The DMPO hydroxyl spin adduct has a characteristic EPR spectrum of a 1:2:2:1 quartet (due to the similar A_N and A_H values) so is easily distinguishable from the other three nitrone spin adducts. A typical spectrum and simulation obtained are shown in Figure 4. The A_N

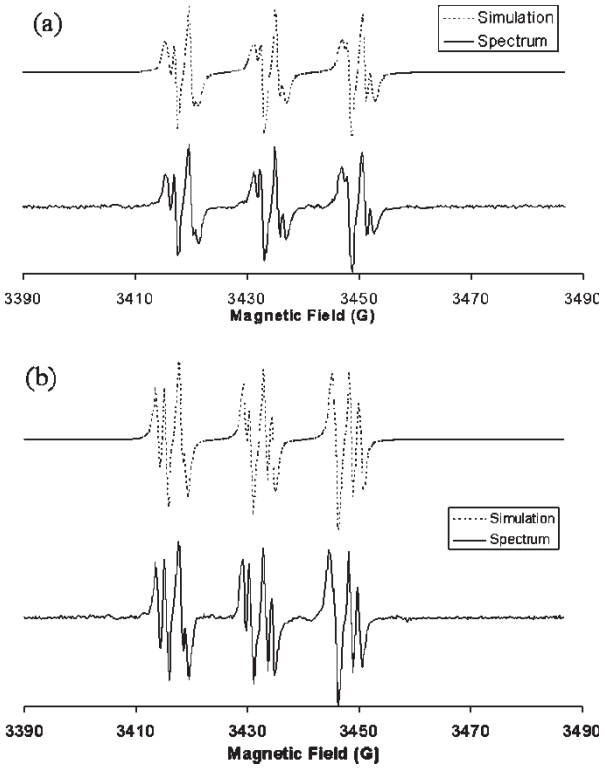

Figure 3. Typical EPR spectrum and simulation obtained for: (a) NXY-059 vs. PBN competitive hydroxyl radical trapping study and (b) S-PBN vs. PBN competitive hydroxyl radical trapping study. Instrument setting: receiver gain 1×10^5, microwave power 2 mW, modulation amplitude 1 G, scan time 21 s, 9 scans.

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Table II. Competitive hydroxyl radical trapping results.

		Number of spin adduct molecules	
		Ti(III)—pH 1.8	Fe(II)—pH 3.2
PBN vs. S-PBN	PBN	8.73×10^{18}	6.87×10^{17}
	S-PBN	7.16×10^{18}	8.99×10^{17}
PBN vs. NXY-059	PBN	1.05×10^{19}	1.45×10^{18}
	NXY-059	5.87×10^{18}	8.99×10^{17}

and A_H values obtained for the DMPO hydroxyl spin adduct ($A_N = 14.97$ G, SD = 0.08 and $A_H = 14.72$, SD = 0.09) were in agreement with those published in the NIEHS database [9]. Within experimental error, similar results were obtained for the three nitrones in terms of the number of molecules of each spin adduct species detected in the presence of DMPO.

Spin adduct lifetime results

The lifetime of the hydroxyl radical spin adduct of each nitrone was investigated by monitoring the EPR response after the flow of reactant solutions was halted. The results obtained are shown in Figure 5. Each hydroxyl radical spin adduct gave a similar decay curve and after 20 s the EPR response of each spin adduct could no longer be detected. In previous work the stability of the hydroxyl radical spin adducts of a range of PBN type spin traps has been studied and the half life shown to vary with the pH and spin trap used, from 2 to 730 s [12].

NMR results

^{1}H NMR studies were performed on the individual spin trap solutions under both reaction conditions and in reactant solution A. The NMR results

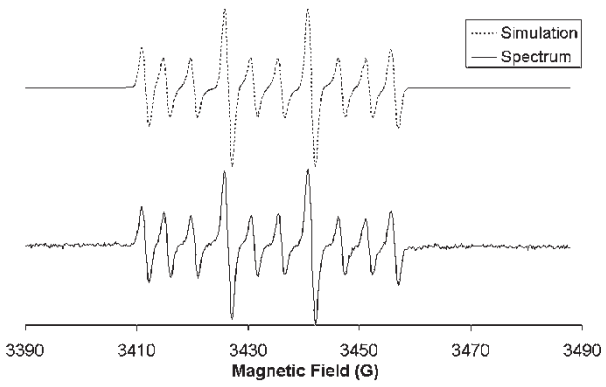

Figure 4. Typical EPR spectrum and simulation obtained for NXY-059 vs. DMPO hydroxyl radical trapping competitive study. Instrument settings: receiver gain 1×10^5, microwave power 2 mW, modulation amplitude 1 G, scan time 21 s, 9 scans.

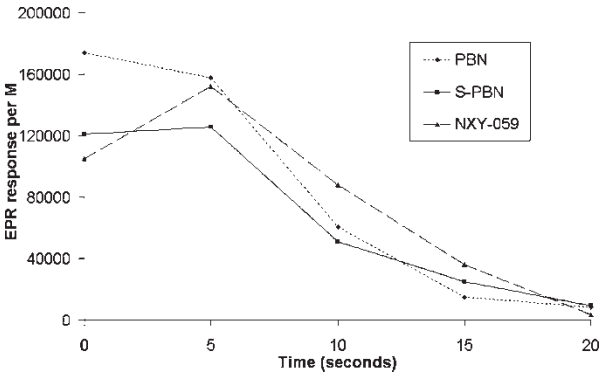

Figure 5. Peak height of the low field peak of the six line EPR spectrum of the hydroxyl radical adduct per mole of spin trap present for each nitrone as a function of the time since the reactant solution pump was switched off. Each point is the mean of three replicates. Instrument settings: receiver gain 1×10^5, microwave power 2 mW, modulation amplitude 1 G, scan time 5 s, 1 scan.

obtained in reactant solution A confirmed that no significant degradation had occurred on a timescale comparable to the entire EPR experiment. In the reaction conditions at pH 1.8 the results showed that on the time scale taken to record an NMR spectrum over 90% of the NXY-059 present had degraded, with only 10% still present as the spin trap species. Similar studies of S-PBN and PBN showed that ~80 and 45% degradation, respectively, of these species had occurred. The NMR results suggested that some degradation of the three spin trap species was also likely to occur during the EPR experiments, although not to the same extent due to the shorter timescale involved. 2D NMR analysis confirmed the identification of the NXY-059 degradation products formed under these acidic conditions, as shown in Figure 6. The same NMR analysis was attempted at pH 3.2 but the presence of iron (II) affected the resolution of the spectra so quantitation was not possible.

Discussion

The EPR spectroscopy results obtained have shown that NXY-059, S-PBN and PBN successfully trap the hydroxyl and methanol radicals. Using a mixing cell resonator with the correct flow of reactant solutions, EPR measurements can be made such that the EPR spin adduct response is independent of the stability of the spin adducts. This configuration allows for

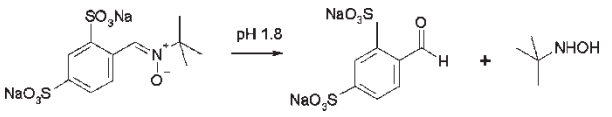

Figure 6. Schematic of degradation of NXY-059 at pH 1.8, as determined by ^{1}H NMR.

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quantitative comparison of the results for different spin traps. However, it is noted that the stability of the spin traps themselves should also be evaluated under the reaction conditions and taken into consideration in quantitative analysis. In this study acidic reaction conditions were used to enhance the yield of radicals formed by chemical reaction. However, this is likely to have had a detrimental effect on the stability of the three nitrones studied, as shown by the NMR results. Ideally to reproduce *in vivo* conditions and enhance the stability of the spin traps the experiments should be performed at pH 7. However, this did not prove possible as the low yield of radicals produced at this pH resulted in weak or non-detectable spin adduct EPR responses (experiments performed at pH 7.3 in 0.1 M phosphate buffer in the presence of 2 mM iron chloride hydrate).

In individual studies NXY-059 was shown to trap more hydroxyl and methanol radicals than the other two nitrones under the conditions studied. The NMR results showed that NXY-059 was the least stable of the three nitrones under the reaction conditions and if this effect could be quantitatively factored into the EPR results it would only enhance the spin trapping ability of NXY-059 compared to PBN and S-PBN under the conditions studied. In competitive hydroxyl radical trapping studies, however, the three spin traps all gave similar results, i.e. the spin trapping ability of each nitrone appeared to be affected by the presence of the second nitrone. This apparent change in the relative spin trap ability of the three nitrones suggests that the diffusion of the spin traps through the reaction mixture to trap the radical in question is an important parameter that should not be over-looked when attempting to quantify radical spin trapping results. The lifetime of the hydroxyl radical spin adduct in these studies was shown to be less than 20 s, which is in agreement with previous studies [12].

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